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THE UNIVERSITY OF ALBERTA

THE MUTAGENIC ACTION OF NITROUS ACID AND ULTRAVIOLET LIGHT ON CONIDIA OF *NEUROSPORA CRASSA*

by

ALBERT DE LEEUW

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Mutagenic Action of Nitrous Acid and Ultraviolet Light on Conidia of *Neurospora crassa*," submitted by Albert de Leeuw, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The amount of conidial inactivation induced in *Neurospora crassa* by nitrous acid (NA) was dependent on the strain used. Reversion rates in three NA-induced tryp-3 mutant strains were non-linear with time, with each showing a definite optimum time under the experimental conditions. Using NA at 50-percent-inactivation dosage, a 16-fold increase in reversion frequency was obtained. NA- and UV-induced reversion rates in the NA-induced tryp-3 strains were in agreement with results and interpretations of other workers.

ACKNOWLEDGEMENTS

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Thanks are extended to numerous members of the Department of Genetics, Mr. P.O.W. Rhynas for his generous aid in the preparation of this manuscript, and to Mr. J. Buffel for help with some of the laboratory work. The writer is also very grateful to Margaret Maclean for her patience and industrious effort in typing the manuscript.

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INTRODUCTION

The identification of deoxyribonucleic acid (DNA), or ribonucleic acid (RNA) as the genetic material has led to a more directed study of the mode of mutagenic action of several chemical mutagens and base analogues. Investigations concerning the mode of mutagenic action of nitrous acid (NA) on the tobacco mosaic virus (TMV) showed that NA was a potent killing and mutagenic agent. The chemical reaction of NA with the bases of DNA and RNA involves deamination of the natural bases of these nucleic acids. Subsequent replication would lead to new base pairing and ultimately to a change in base-pair sequences. Viruses and bacteria have been extensively used in these early studies.

The following project was initiated in order to investigate the mutagenic action of NA upon *Neurospora crassa*, an Ascomycete, which has DNA as its genetic material. The study was carried out with the multi-nucleate and haploid conidial spores.

The *tryp-3* locus, which controls the synthesis of the enzyme tryptophan synthetase, was selected for this investigation. Several interesting properties of this enzyme are known and these will be referred to later.

The work discussed in this paper consists of the following parts:

(i) the induction of tryptophan auxotrophs by NA;

(ii) the mode of inactivation of *Neurospora crassa* conidia by NA; and

(iii) a study of the back-mutation rates induced by ultraviolet (UV) and by NA in the original NA-induced mutant strains.

LITERATURE REVIEW

A. Induction and Inactivation of Mutations by Nitrous Acid

The mutagenic action of NA was first reported by GHAN and STEINBERG in 1939. They grew *Aspergillus niger* on a medium containing 0.2% $NaNO_2$. Under nitrite stimulation the same stock culture of *Aspergillus niger* produced, in successive experiments, the same type of morphological mutants. Later STEINBERG and THOM (1940 a, b) also obtained NA-induced morphological mutants in *Aspergillus niger* and *Aspergillus amstelodami*. The mutagenic action of NA at that time was ascribed to the deamination of free amino groups of proteins in the mold. This led STEINBERG and THOM (1940 a) to use d-lysine, which was known to associate in one way or another with free amino groups in proteins, as an agent for back mutation. These experiments were only partially successful.

SIDDIQI (1962) found NA to be a potent killing and mutagenic agent for spores of *Aspergillus nidulans*. The dose-effect curves for induction of phenotypically distinguishable suppressors of methionine requirement were non-linear in two of the three cases investigated. Mixed colonies were found when color mutants were studied. The interpretation given was that at the time of treatment more than one genetic strand may have been present. The survival curve was found to be exponential, with a distinct shoulder near the origin.

SCHUSTER and SCHRAMM (1958) studied the inactivation of RMV-RNA* by NA. They showed that TMV-RNA is inactivated by NA according to first-order kinetics when NA is present in an excess amount at 20° C and in a pH range 4 - 4.5. In the absence of NA the TMV-RNA was shown to be stable over a long period of time in this pH range. It was suggested that deamination of certain RNA bases may result in mutation rather than inactivation.

In 1958 GIERER and MUNDRY treated both the intact TMV and its isolated RNA with NA. They studied the production of necrotic lesions on a tobacco variety on which the untreated strain produces chlorotic lesions only. The material treated with NA (50% inactivation) gave a 20-fold increase of necrotic lesions when compared with the same number of untreated infectious particles. The production of mutants followed a single-hit curve. On the basis of this information they concluded that the alteration of a single base leads to mutation. The mutants proved to be genetically stable. Inactivation by, as well as the mutagenic action of, NA upon the bacterial phage T4 in vitro was demonstrated by TESSMAN (1959), HARM (1960) and BAUTZ-FREESE and FREESE (1961). BAUTZ-FREESE and FREESE (1961) demonstrated that NA could increase reversion rates in most mutants which showed spontaneous back mutations and had been induced by 5-bromouracil, 2-aminopurine and NA. However, a number of spontaneous mutants did not show any induced reversion by NA. The reversion rates followed a single-hit curve, indicating that the deamination of a single base can

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* Tobacco mosaic virus RNA

cause a mutation; this fact had also been demonstrated by SCHUSTER and SCHRAMM (1958). The reversion rates of different mutants varied up to a factor of 100.

BENZER (1961) also studied the induction of mutants in phage T4 and found that at 2% survival the viable phages showed 0.4% "r" mutants and mottled plaques. When these mutants were mapped it was found that the NA-induced rII mutants had a different "spectrum" and mutational "hot spots" than spontaneous and otherwise induced mutants.

LITMAN and EPHRUSSI-TAYLOR (1959) treated DNA isolated from Diplococcus pneumonia strains resistant to streptomycin, aminopterin, and optochin with NA. They found inactivation of transformation of these resistance markers into susceptible strains. The inactivation curves were drawn to follow an exponential decrease of transformation to resistance. When DNA isolated from susceptible strains was treated with NA, a definite increase in transformation to resistance was observed. This was ascribed to the mutagenic action of NA upon the transforming DNA treated in vitro.

LITMAN (1961) also found inactivation when transforming-DNA of Diplococcus pneumonia was treated in vitro. She not only found inactivation of markers but also a decline in uptake by the recipient cells of DNA treated with NA. When the induction of mutations to drug resistance was studied, the maximum number of mutations was found to take place at a low level of inactivation, the mutants were found to be stable. Attempts to induce reversions

in some uracil-requiring strains were unsuccessful, but the origin of these mutants was not given.

KAUDEWITZ (1959 a, b) studied the mutagenic action of NA on *Escherichia coli* cells *in vivo*. His work indicated that the number of NA-induced mutants only depends on the duration of incubation in NA and not on the physiological state of the cells. This latter information was held as an indication that NA induces auxotrophic mutants by a direct action on the DNA of the bacterial cell. Seventy to eighty percent of the mutants showed mixed colonies. The induction of auxotrophic mutants showed quadratic kinetics indicating that two hits are necessary for the expression of a mutation in *Escherichia coli*.

In vitro inactivation and induction of mutation in transforming DNA of *Bacillus subtilis* by NA was studied by ANAGNOSTOPOULOS and CRAWFORD (1961). They found high inactivation of a histidine marker and a good yield of tryptophan auxotrophs.

The *in vitro* effect of NA on transduction by SALMONELLA phage P22 was studied by ADYE (1962). The phage survival during treatment decreased exponentially with single-hit kinetics. Most of the histidine-requiring mutants obtained in this study were found to have an incomplete genetic block of the biochemical requirement.

BARNETT and DE SERRES (1962) and CASE (1962) studied the back-mutation properties of NA-induced mutants in *Neurospora crassa*. They found that the NA did increase the back-mutation rates of some of the NA-induced mutants.

GUTZ (1961) induced _ad-7_ mutants in _Schizosaccharomyces pombe_ by NA. He found that about 50% of his mutants were temperature sensitive.

All the above-mentioned authors had no doubt that their work did show a mutagenic action of NA on the DNA or RNA either by _in vivo_ or _in vitro_ treatment. However, STUY (1961, 1962) did not find any evidence for the mutagenic action of NA upon the transforming DNA of _Hemophilus influenzae_. He also found the frequency of transformation of drug-resistance markers reduced due to failure of inactivated markers to be incorporated into the recipient genome.

HORN and HERRIOTT (1962) confirmed the work of STUY. They did not find new antibiotic markers when they treated native _Hemophilus influenzae_ DNA with NA. However, treatment of heat-denatured DNA of _Hemophilus influenzae_, followed by renaturation, gave a large number of transformable antibiotic-resistance markers. Most of the NA-induced markers showed resistance to the exposure of NA.

LUZZATI (1962) confirmed the work of HORN and HERRIOTT on the differential mutagenic action of NA upon native and heat-denatured DNA of _Hemophilus influenzae_ transforming principle.

B. Action of Nitrous Acid

I. Deamination

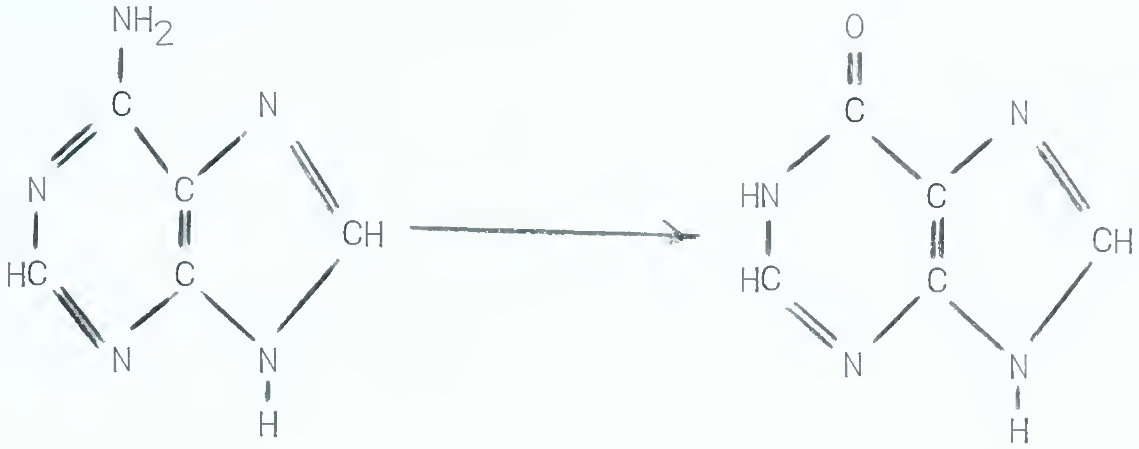
The deamination of the purines and pyrimidines by NA was first studied by VAN SLYKE (1911). He showed that NA oxidatively deaminates the NH_2 groups of amino acids, purines and pyrimidines. Under identical conditions NA reacts more rapidly with the amino groups of free amino acids than with those of free guanine, adenine and cytosine. The reaction of NA with the amino groups occurs in the following overall manner:

$$R.NH_2 + HNO_2 \longrightarrow ROH + H_2O + N_2$$

Amino groups in the proteins are deaminated only to a very slight extent as NA does not deaminate the amino groups involved in peptide linkages. Only the free amino groups of proteins were shown to be oxidatively deaminated.

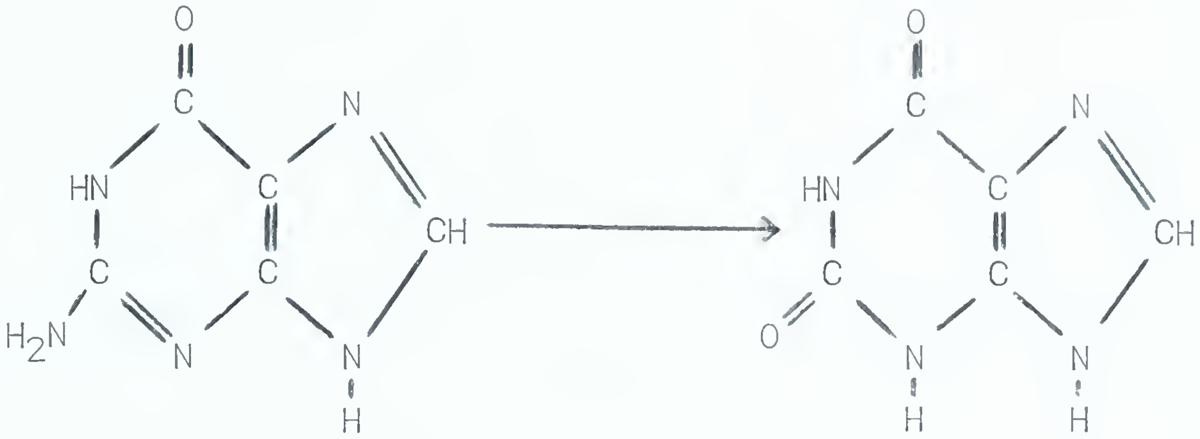
The rate of deamination of nucleic acid bases in the DNA-polymer is much slower than for free purines and pyrimidines (BREDERECH et al. 1938).

The deamination of TMV-RNA by NA was studied by SCHUSTER and SCHRAMM (1958 a, b). The isolated RNA was treated with an excess of NA at pH 4 - 4.5. It was found that the inactivation of the RNA was not accompanied by a degradation of the polynucleotide chain. The only observable alteration was found in the RNA bases, which were deaminated. The reaction products were isolated and these consisted of:



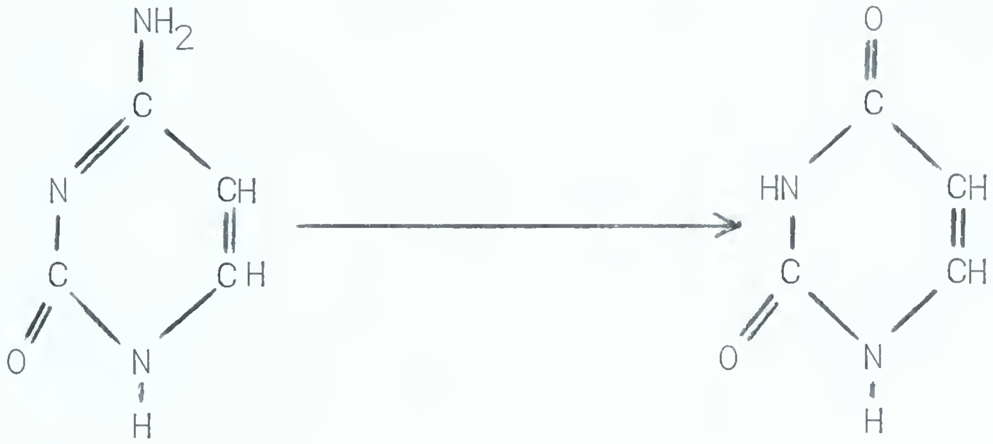
Adenine (y-amino-purine)

Hypoxanthine (6-oxypurine)



Guanine (2 amino-6-oxypurine)

Xanthine (2.6. dioxypurine)



Cytosine (2-oxy-6-aminopyrimidine)

Uracil (2.6 dioxypyrimidine)

After Davidson in The Biochemistry of the Nucleic Acids (Methuen's Monograph on Biochemical Subjects) 1961.

A linear increase of the reaction products with time was obtained. RMV-RNA incubated at pH 4 - 4.5 without NA did not show any increase of the reaction products. TMV-RNA is a single-stranded

molecule and contains 6000 nuclear bases; one hit per 3000 bases resulted in the loss of activity of the entire molecule.

The relative rate of deamination of phage T2-DNA bases was studied by VIELMETTER and SCHUSTER (1960). Under their conditions they found this to be adenine (A) : cytosine (C) : guanine (G) = 1 : 6.3 : 16. From their inactivation work accompanying this study they concluded that deamination of A, C and G can be lethal.

When LITMAN (1961) studied the deamination sequence of *Pneumococcus* DNA she found that 10% of the guanine deaminated very rapidly, and the remainder deaminated at a slower rate but still five times faster than the deamination rate of adenine. The deamination of cytosine begins after a lag period during which 15% of its base partner, guanine, has been converted to xanthine. This was held to indicate that the cytosine amino group can be deaminated only after its base partner has been deaminated. After this lag period cytosine is deaminated twice as fast as adenine. These results are in good agreement with those obtained by VIELMETTER and SCHUSTER (1960). At 99% inactivation of the transforming properties of the DNA, only the deamination of guanine and adenine were detectable; at this time 10 - 15% of the guanine and 1 - 2% of the adenine had been converted to xanthine and to hypoxanthine respectively.

2. Induction of cross linkages

The absence of mutagenic activity of NA upon transforming *Hemophilus influenzae* DNA treated *in vitro* was shown by STUY (1961), and HORN and HERRIOTT (1962). This observation is in conflict with the results generally obtained. GEIDUSCHEK (1961-1962) treated *Hemophilus influenzae* DNA with NA and found that it showed a greater resistance to acid denaturation than did the DNA of *Diplococcus pneumonia* and *Bacillus subtilis*. The denatured DNA also renatured more extensively when it was brought back slowly to neutral pH. These results were interpreted as being an indication of the formation of covalent cross links between the two strands of the DNA double helix. In *Hemophilus influenzae* the above discussed covalent cross links in its transforming DNA could explain:

(i) the greater resistance to acid denaturation by holding the double helix together;

(ii) the greater ability of renaturation as the cross links can act as "nucleation centers" during renaturation; and

(iii) the lack of the integration of markers into the recipient genome as found by STUY (1961).

The cross links would prevent the separation of the double helix during replication and so prevent the incorporation of genetic information into the recipient genome. No indication is found as yet why *Hemophilus influenzae* DNA differs from *Bacillus subtilis* in its reaction towards NA. When heat-denatured DNA of *Hemophilus influenzae* was treated with NA and then renatured, a definite increase of mutations occurred (LUZZATI 1962, HORN and HERRIOTT 1962).

This would indicate that under conditions where cross linkages are excluded the abnormal behavior of Hemophilus influenzae DNA is also absent.

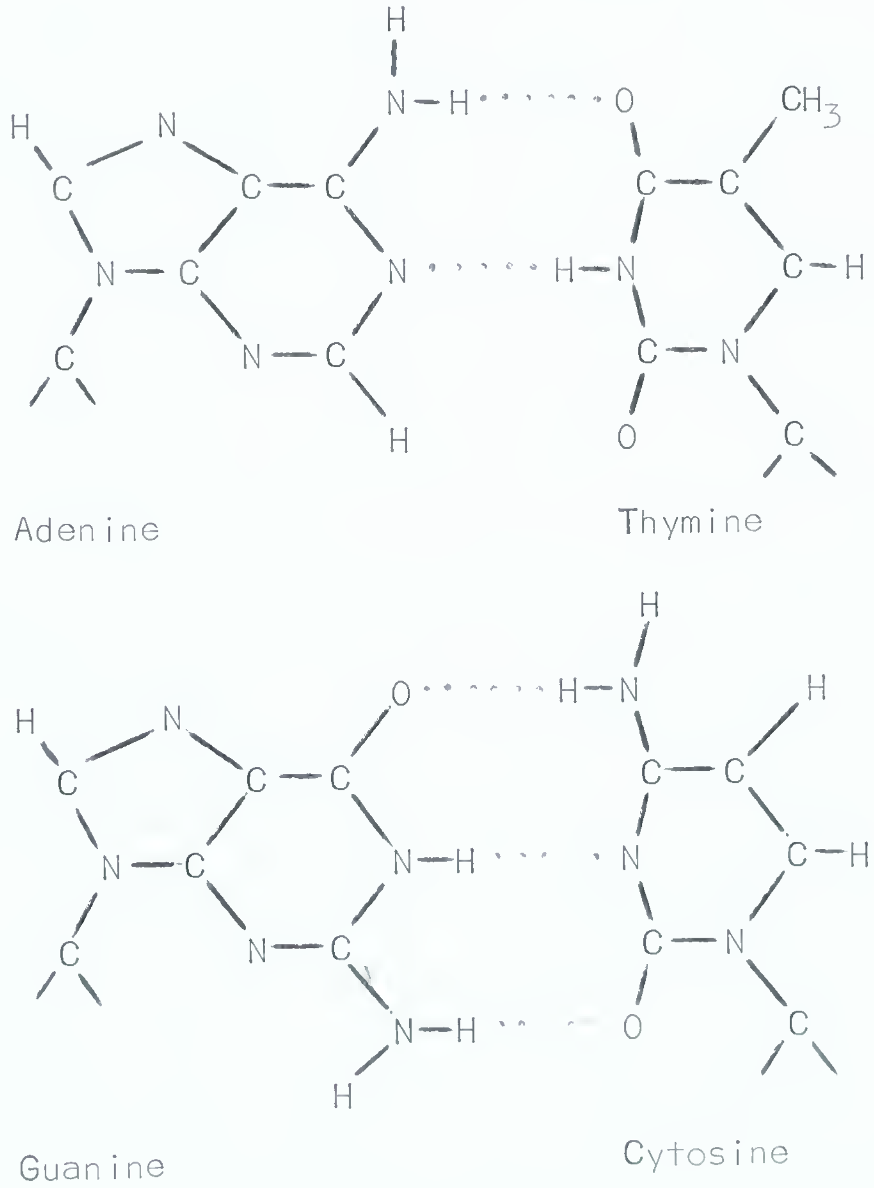
3. Induction of deletions

Induction of large deletions by NA in phage T4 was shown by TESSMAN (1962). These T4 mutants have preferential end-points. Therefore they are probably produced by so-called "stumbling blocks" during replication. It has been visualized that the stumbling block is circumvented by a looping of a region of the parental DNA-strand, whereas replication continues normally further down the strand. Deoxyxanthine triphosphate cannot serve as a substrate for *in vitro* DNA synthesis (BESSMAN *et al*. 1958). It might be that xanthine also interferes with the *in vivo* replication of the DNA. VIELMETTER and SCHUSTER (1960), studying inactivation and base changes induced by NA, concluded from their studies that the deamination of guanine to xanthine might interfere with DNA replication and lead to lethal consequences. TESSMAN (1962) considers that this change might interfere with the DNA replication. If a single guanine deamination leads to this event the occurrence of deletions among the NA-induced mutants should be a general phenomenon with a high occurrence, as guanine is the base which is most readily deaminated (LITMAN 1961, SCHUSTER and SCHRAMM 1960). Evidence for such a point of view, however, is still lacking. The covalent cross linkages which occur in Hemophilus influenzae (GEIDUSCHEK 1962) might occur less frequently in phage-T4 DNA treated with NA and are more easily visualized as a stumbling block

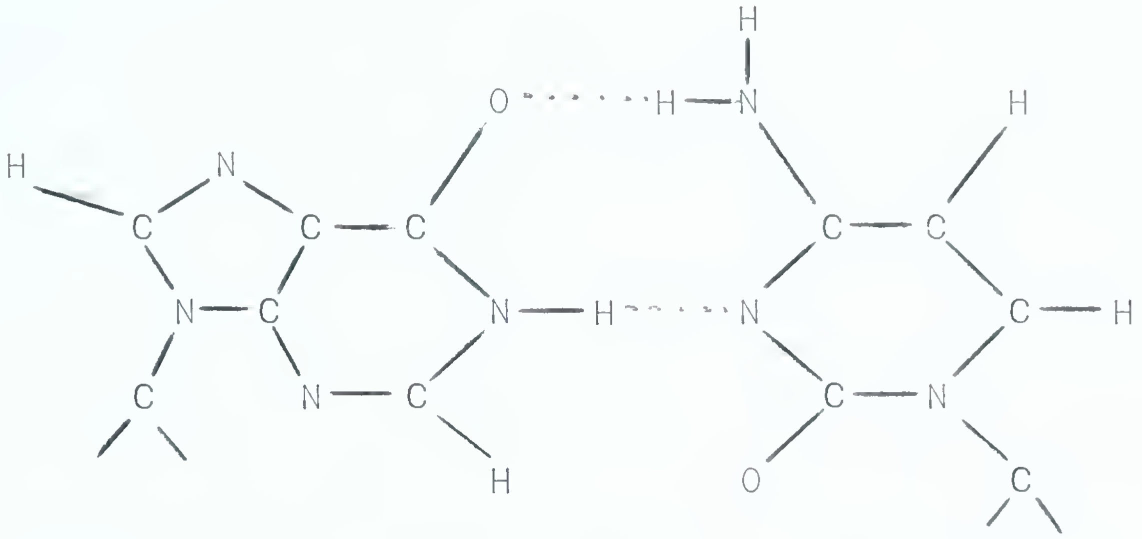
for DNA replication. In any event the mutagenic action of NA seems to depend on phenomena other than base changes alone.

C. Base-Pair Changes Resulting from Base Deamination

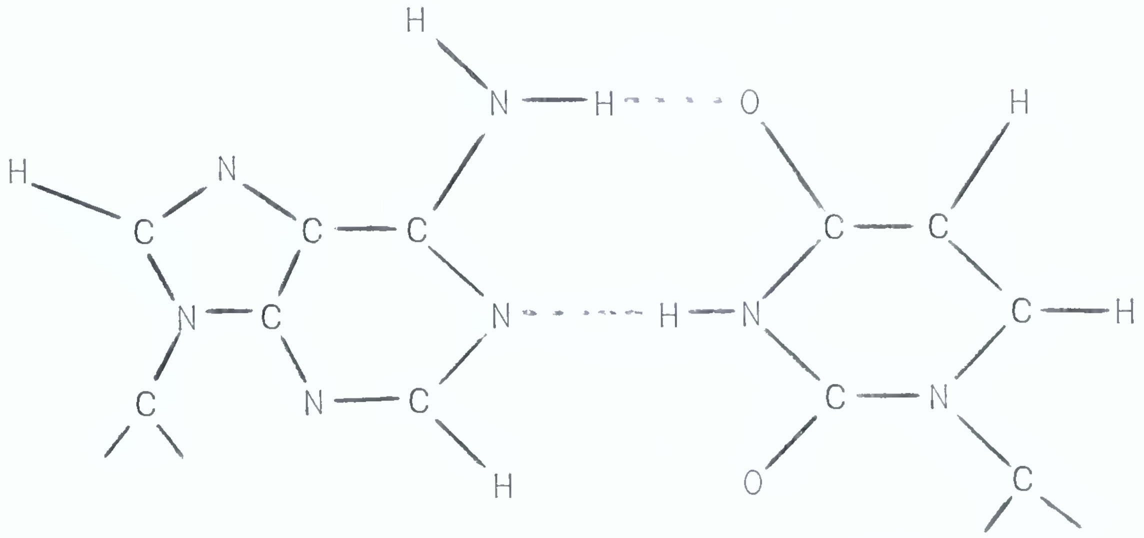
The effect of deamination of the bases lies in the fact that after deamination the hydrogen-bonding properties of the bases are changed. Adenine deaminates to hypoxanthine which now pairs with cytosine instead of with thymine. Cytosine deaminates to uracil which now pairs with adenine instead of with guanine. Guanine when deaminated becomes xanthine which still pairs with cytosine, but by one hydrogen bond less.



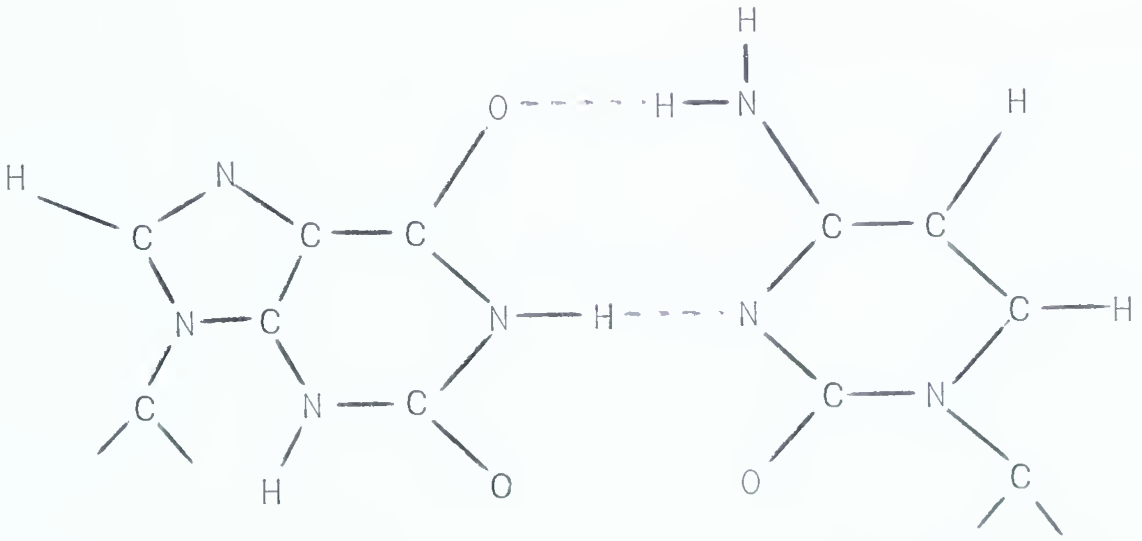
Base pairing in the Watson-Crick structure. Hydrogen bonds are shown dotted. One carbon of each sugar is shown. (After Watson and Crick, Cold Spring Harbor Symposia Quant. Biol., Vol. 10, 1953.)



Hypoxanthine Cytosine



Adenine Uracil

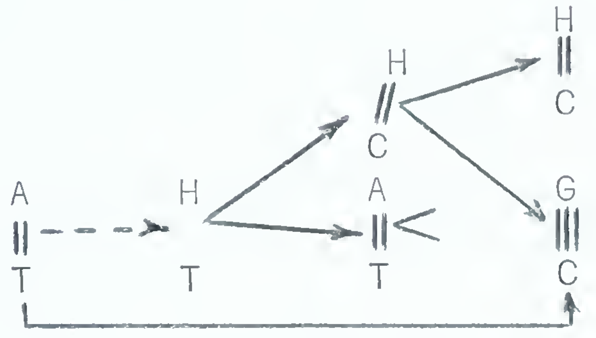


Xanthine Cytosine

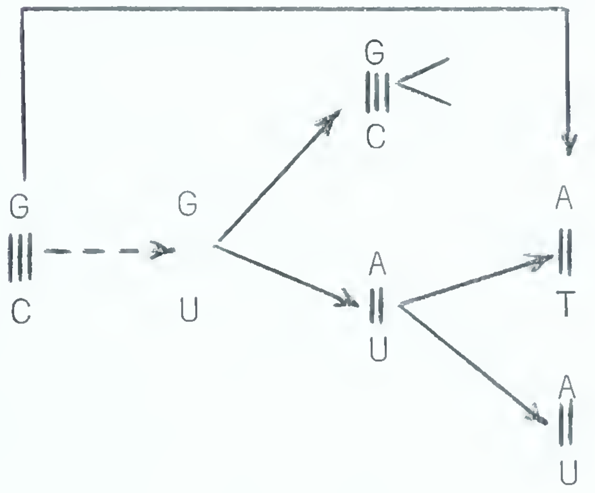
Base pairing of deaminated bases.

(cf. footnote page 13)

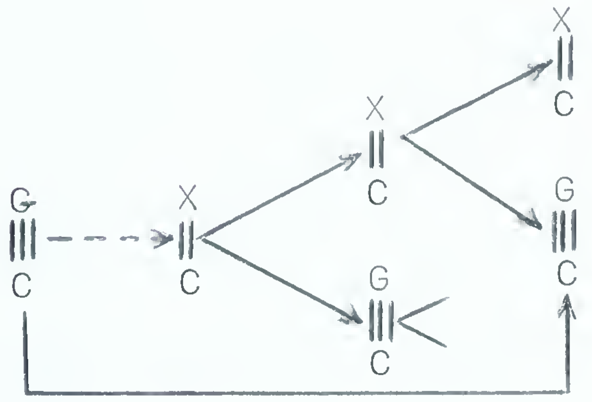
Assuming that during DNA replication the two strands of the double helix separate, with each forming its own complementary strand, then the change of base pairing properties will have the following consequences:



Deamination of Adenine



Deamination of Cytosine



Deamination of Guanine

As shown by the long arrows in the above scheme, after deamination of adenine and subsequent replication, an $A = T$ pair changes to a $G \equiv C$ pair; similarly, a $G \equiv C$ pair, after deamination of cytosine, changes into an $A = T$ pair. The deamination of guanine in the $G \equiv C$ pair does not lead to this kind of base-pair change.

The only base-pair changes that could arise in this way are transitions, this means one purine is replaced by another purine and one pyrimidine by another pyrimidine (BAUTZ-FREESE and FREESE 1961). According to this scheme only the deamination of cytosine and adenine are mutagenic and the base pair changes induced are reversible:

$$\begin{matrix} A & & G \\ \| & \longleftrightarrow & ||| \\ T & & C \end{matrix}$$

The rate of deamination of cytosine is 6.3 times as fast as that of adenine (VIELMETTER and SCHUSTER 1960). This should mean that the base pair change $G \equiv C$ to $A = T$ should occur more often than the reverse change. BENZER (1961), studying the rII region of phage T4 and GUTZ (1961) studying the *ad-7* region of *Schizosacchoromyces pombe*, both found that NA induces mutations at hot spots along the loci. These hot spots are different from those induced by other mutagenic agents. This seems to indicate either that the mutation rate depends not only on the bases involved in deamination but also on the neighbouring sites, or that the hot spots contain a high concentration of $A = T$ base pairs. Another factor which might influence the apparent mutation rate is the ultimate effect that the base change has on the protein. YANOFSKY and HENNING (1962) found that four different amino acids could have the same position in the enzyme tryptophan synthetase and that in all cases the protein would be still active. It was reasoned that if the NA-induced mutations would lead to the incorporation of different amino acids at different loci inactivating the function of the protein, then such mutations

would not be expressed. If, however, an auxotrophic mutant is induced at these loci then there is more than one possibility for back mutation and the back-mutation rate found would not be a true indication of the base changes induced at the changed base pair only, but also of the base changes induced in the neighbouring base pairs coding for the same amino acid. These phenomena would lead to reduced forward mutation rates and to an increase in back mutation rates in certain spots of the genetic material. These side effects of the protein on the expression of the mutations could explain the up-to-100 fold differences in back-mutation rates induced by NA between the various NA-induced mutants which show spontaneous back mutation as found by BAUTZ-FREESE and FREESE (1961). This is in contrast to the six-fold difference found in the *in vitro* deamination rates of the bases adenine and cytosine. However, the mutants showing the highest mutation rates have most likely a G $\equiv$ C pair at the mutant site.

D. Mixed versus Pure Mutant Colonies

MUNDRY and GIERER (1958) showed that the inactivation and induction of mutations followed a single-hit curve for TMV-RNA. KAUDEWITZ (1958) on the other hand showed that the inactivation and the induction of pure auxotrophic colonies followed a two-hit curve in *Escherichia coli*. On first sight these data seem to be contradictory, however, it has to be remembered that in these two organisms a difference exists in the carriers of the genetic information. TMV-RNA is a single-stranded structure (SCHUSTER 1960), while the DNA in *Escherichia coli* is a double-stranded structure.

According to the scheme outlined on page 15 one expects to obtain mixed colonies when only one strand is hit, because after replication the second strand will yield the unchanged base pair, giving rise to wild-type offspring. Thus for the induction of pure auxotrophic colonies in this case two hits are needed, one for the induction of a mutation, the other for the inactivation of the complementary strand. KAUDEWITZ _et_ _al_. (1963) studied the rates of induction of mixed and pure mutant colonies and found that this followed the expected ratio. SIDDIQI (1962), studying the appearance of color mutants in _Aspergillus_ _nidulans_, found sectored colonies. This finding also indicates that one strand can be changed while the other remains unchanged. Mixed colonies are thus most likely the result of a mutagenic hit in one strand while the other strand is lethally affected. Pure mutant colonies are the result of at least two hits, one inducing the mutation the other inactivating the opposite strand. This would explain the difference in inactivation rates between TMV and _Escherichia_ _coli_, because TMV-RNA being single stranded would need only a single hit to be either mutated or inactivated.

E. Unstable Mutants

ZAMENHOF (1961), studying the induction of lactose fermenting (_Lac_$^+$) mutants in _Escherichia_ _coli_ by NA found whole mutant colonies and mixed colonies. Upon restreaking some of the mixed colonies gave 60% stable, unmixed colonies and 40% mixed colonies. These mixed colonies upon restreaking yielded 80% stable wild type colonies, 16 - 18% stable mutant colonies and 2 - 4% mixed colonies. This process could be repeated indefinitely and

the properties of the mixed colonies remained the same. When the mixed colonies, however, were incubated in a medium selective for *lac*$^+$ cells only, the unstable cells were not eliminated by this type of medium, which indicated that the *lac*$^+$ genome had been unstabilized. Following *in vivo* treatment with NA, unstable auxotrophs have been obtained in both *Escherichia coli* and *Bacillus subtilis*. These mutants showed a back-mutation rate in the order of 1 per 10^4 to 4 per 10^2 cells per generation. ZAMENHOF defines the term "gene unstabilization" as a phenomenon in which a gene mutates to a higher spontaneous mutation-rate. "Unstable genes" were arbitrarily designated as those having a spontaneous mutation-rate higher than 1 per 10^4 cells per generation. However, no attempt has been made to localize these unstable genes. It is thus impossible to say whether the mutation is at the nominal locus itself, or whether it is at another so-called "mutator" locus. Mutator loci have been found in *Drosophila* (DEMEREC 1941), in *Zea* (RHOADES 1941) and in bacteria (LEDERBERG 1952). LEDERBERG also found among his unstable mutants some which contained the unstable factor at the nominal locus itself. Unstable genes which occurred spontaneously (LEDERBERG 1952) have been found, whereas others have been induced by 5-bromouracil (ZAMENHOF, DE GIOVANNI and GREER 1958). All other mutants found were reported to be stable, showing normal spontaneous back-mutation rates. These results seem to indicate that "gene unstabilization" is a phenomenon which is not due to a specific action of NA alone.

F. The Tryptophan Pathway

The pathway for the production of tryptophan in *Neurospora crassa* and *Escherichia coli* has been studied very extensively by genetical and biochemical means. The *tryp-3* locus has been selected, because the gene product, the enzyme tryptophan synthetase, has very many interesting properties and hence the mutants produced can be used for further biochemical studies. Reviews have been written on the tryptophan pathway and on the enzyme tryptophan synthetase by YANOFSKY (1960) and by BONNER (1960), and the biochemical pathway for tryptophan as outlined on page 21 is adapted from these two publications. The genetic blocks indicated therein refer to the abbreviations of the genetic loci in *Neurospora crassa* controlling the enzymes involved in the biochemical blocks.

Neurospora crassa has the following five loci, which when rendered inactive, will make the organism tryptophan dependent (Neurospora Information Conference):

(i) *Tryp-1*, linkage group IIIR, requires indole or tryptophan;

(ii) *Tryp-2*, linkage group VIR, requires anthranilic acid, indole or tryptophan;

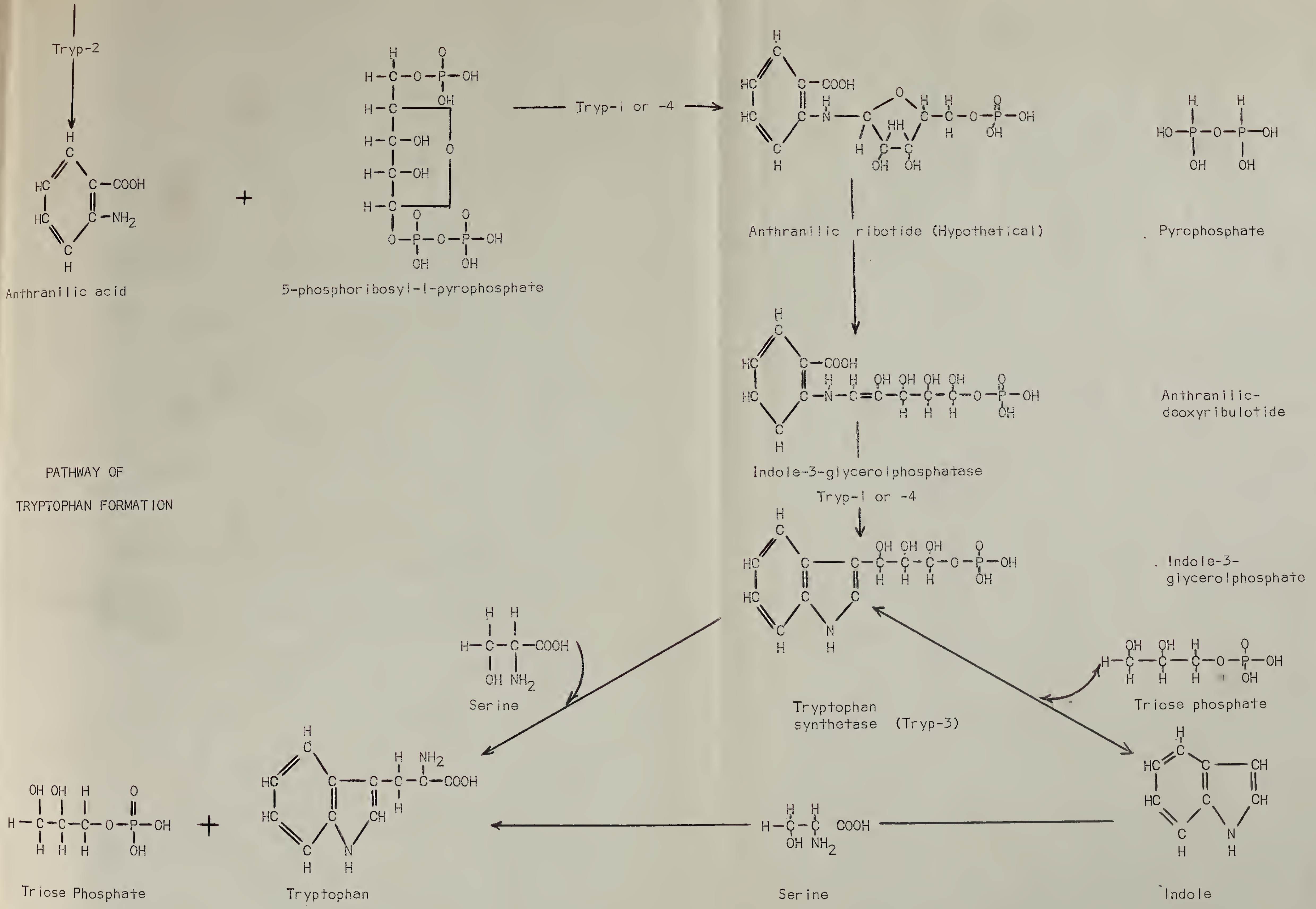
(iii) *Tryp-3*, linkage group IIR, requires tryptophan and some mutants can use indole;

(iv) *Tryp-4*, linkage group IVR, requires tryptophan or indole; and

(v) *nt*., linkage group VIIIR, can use either nicotinic acid or tryptophan and is involved in the pathway of nicotinic acid production.

PATHWAY OF

TRYPTOPHAN FORMATION


Tryp-2
Anthranilic acid
5-phosphoribosyl-1-pyrophosphate
Tryp-1 or -4
Anthranilic ribotide (Hypothetical)
Pyrophosphate
Anthranilic-deoxyribulotide
Indole-3-glycerolphosphatase
Tryp-1 or -4
Indole-3-glycerolphosphate
Serine
Tryptophan synthetase (Tryp-3)
Triose phosphate
Triose Phosphate
Tryptophan
Serine
Indole

After Yanofsky (1960) and Bonner (1960)

It is obvious that we cannot select the _tryp-3_ mutants (also called _td_-mutants by BONNER's school) by growth requirement alone since mutations at three different loci show the same requirements. The genetic test of linkage was used to determine the mutant's genetic block. The morphological marker _fluffy_ is located very closely to and proximal of the _tryp-3_ locus. _Fluffy_ does not produce any macro-conidia and only a few micro-conidia are formed. This morphological mutant can easily be distinguished from the phenotypically wild-type strain. This mutant was used for the determination of the location of the _tryp-3_ mutants.

MATERIALS AND METHODS

I. Media

The following media were used:

Minimal medium (VOGEL 1956)

In 750 ml distilled water dissolve successively with stirring at room temperature:

Na_3 citrate .5-1/2 H_2O	150 gm
KH_2PO_4 (anhydrous)	250 gm
NH_4NO_3 (anhydrous)	100 gm
$MgSO_4$. 7 H_2O	10 gm
$CaCl_2$. 2 H_2O	5 gm
Trace elements solution	5 ml
Biotin solution	2.5 ml.

Resulting total volume is about 1000 ml.

2 ml chloroform is added as a preservative.

Stock solution is 50 times normal strength.

To obtain minimal medium dilute stock solution 50 times and add a carbon source (e.g. 20 gm sucrose per liter).

Trace element solution

Dissolve in 95 ml distilled water successively at room temperature with stirring:

Citric acid. 1 H_2O	5.00 gm
$ZnSO_4$. 7 H_2O	5.00 gm
$Fe(NH_4)_2(SO_4)_2$. 6 H_2O	1.00 gm
$CuSO_4$. 5 H_2O	0.25 gm

$MnSO_4 . 1 H_2O$	0.05 gm
H_3BO_3 (anhydrous)	0.05 gm
$Na_2MoO_4 . 2 H_2O$	0.05 gm

The resulting total volume is about 100 ml.

1 ml chloroform is added as a preservative and the trace element solution is stored at room temperature.

Biotin solution

This solution is prepared by dissolving 5.0 mg biotin (MERCK) in 50 ml distilled water. The resulting solution is dispersed in test tubes and stored in the frozen state.

Complete medium

Supplement normal minimal medium with:

0.5% yeast extract (Difco)

0.5% casein (N-Z Case)

1.0% sucrose

1.0% glycerol

1.5% agar (Difco Bacto-Agar)

Crossing medium (WESTERGAARD and MITCHEL (1947)

A synthetic medium favoring sexual reproduction:

K_2HPO_4	0.35 gm
KH_2PO_4	0.65 gm
$MgSO_4 . 7 H_2O$	0.50 gm
NaCl	0.10 gm
$CaCl_2$	0.10 gm
Biotin	5
Trace elements	1.0 ml

KNO_3	1.0 gm
Distilled water	1000.0 ml
*Sucrose	20.0 gm
*Agar	15.0 gm

* Add later to avoid cooling of sugars.

Final pH 6.5

Stock solutions were made two times normal concentration.

Supplemental medium

l-tryptophan	50 mg/liter
Inositol	25 mg/liter
Indole	50 mg/liter
Anthranilic acid	50 mg/liter
Nicotinic acid	50 mg/liter

Sorbose plating medium

Sorbose is used as a carbon source in the medium when colonial growth is wanted. It is sterilized separately to prevent caramelization.

1.5% sorbose

0.2% glucose

0.2% glycerol

2.0% agar

1x normal minimal medium

2. Materials

Strain 74-A (the St. Lawrence Standard 74-A) and the fluffy strains fl^--A and fl^--a were kindly supplied by Dr. J. Weijer. The inositol-requiring strain $inos^-$89601-A was obtained from Dr. E.L. Tatum.

The various UV-induced tryp-3 mutants were obtained from W.E. Barratt of the Fungal Genetic Stock Center and the code number used in the discussion are the same as those reported in the literature.

3. Methods

A. Induction of mutants by nitrous acid

The following method, which is a slight modification of the one used by KØLMARK and DE SERRES (private communication), was used for the induction of mutations and for the study of the inactivation of conidial spores by NA.

(I) Preparation of conidial suspension

Cultures of wild-type strain 74-A or of any other strain used were grown on minimal synthetic medium, supplemented with the necessary requirements if any were needed, for six to seven days at 25° C. Conidial suspensions were made in cold sterile water filtered through glass-wool filters, washed two times with pH 4.6 (0.1 M) acetate buffer and adjusted to 1.11×10^7 conidia per ml.

Acetate buffer (0.1 M, pH 4.6):

2.92 ml glacial acetic acid

4.1 gm sodium acetate (anhydrous)

1 liter distilled water

(2) Start of treatment

To 9 ml of conidial suspension (1.11×10^7 conidia/ml in pH 4.6 acetate buffer) 1 ml of $NaNO_2$ (10 times the molarity of the one wanted) was added and incubated at the desired temperature in a constant-temperature water bath.

(3) Termination of treatment of conidial suspension

Aliquots were taken out of the treatment vessels and diluted at least 10 times in cold phosphate buffer (pH 7) to stop the reaction. For induction of mutants these were then centrifuged, decanted and washed two times with Phosphate buffer at pH 7. For the determination of survival they were then diluted to 10^3 conidia/ml and 0.1 ml of this solution was plated on minimal sorbose medium.

Phosphate buffer, pH 7:

0.02 M KH_2PO_4	2.722 gm/liter
0.03 M Na_2HPO_4	4.260 gm/liter
0.02 M NaCl	1.169 gm/liter

B. Screening for mutants

Two methods for screening out the wild types were employed:

(1) Filtration enrichment (WOODWARD, DE ZEEUW and SRB 1954)

After treatment the conidia were suspended in a flask containing 500 ml minimal liquid medium. These cultures were grown with forced aeration at room temperature. Every 24 hours this

suspension was poured through glass wool under sterile conditions to remove germinated conidia. Conidia left in this flask after three days of incubation were centrifuged out and plated on minimal sorbose medium supplemented with tryptophan and the plates were incubated at 30° C.

(2) Inositol-less method (LESTER and GRASS 1959)

This method takes advantage of the reduced rate of death of germinating conidia of an inositol-requiring strain when a second mutation is induced. Inositol-requiring strain 89601-A was used during induction of mutants to minimize the number of reversions to prototrophy. The treated conidia were suspended in minimal medium containing 1% sucrose and 0.25 μg inositol per ml, and incubated for 6 hrs at 25° C at 1×10^6 conidia/ml to minimize the loss of induced mutations due to delay of phenotypic expression. The suspension was shaken vigorously to prevent heterocaryosis. At the end the suspension was filtered through glass wool, washed 3 times with H_2O and adjusted to $10^7 - 10^8$ conidia per ml. One ml aliquots of this suspension were plated in 12 ml sorbose medium without supplements. The plates were incubated for 3 days at 30° C, then overlayed with a "4X" complete sorbose medium containing:

5 mg inositol per liter; and
200 mg l-tryptophan per liter.

Following this the plates were incubated at 30° C.

The colonies which appeared after 2 - 3 days on these plates were transferred to small tubes containing complete medium. After the cultures became established on this medium, they were

tested out on minimal liquid medium. If no growth was observed then the culture was given an isolation number and later tested for growth on minimal medium and on minimal medium supplemented with l-tryptophan. If the cultures did grow on minimal medium supplemented with tryptophan and not on minimal medium they were then subcultured. These cultures were tested to see if their growth requirement could be met by either anthranilic acid, indole, or typtophan. Cultures whose growth requirement could be met by tryptophan and indole only, were crossed with $\underline{fl^-a}$ to determine if the mutation was located in the linkage group IIR (chromosome II, right of centromere).

C. Spontaneous reversion test

Conidial suspensions were made as outlined under the heading: "Induction of Mutations," adjusted to 1×10^7 conidia per ml, diluted in 50 ml of minimal sorbose medium and distributed over 10 plates. After 3 and 6 days of incubation at 30° C the number of wild-type colonies was counted. A small sample from the initial conidial suspension was taken, diluted down to 1×10^3 conidia per ml and four 0.1 ml samples were then spread out over complete sorbose plates to determine the viability of the conidia plated.

D. Ultraviolet light and nitrous acid induced reversion test

The method used for this test is similar to that mentioned under the heading "Spontaneous reversion test." After a small sample is taken for determination of the viability of the conidia before irradiation, the remainder was diluted down to 10^6 conidia

per ml. Fifteen ml of this suspension was irradiated with UV (20 watt General Electric Germicidal Lamp) for 35 seconds at a distance of 20 cm. Of this irradiated suspension 10 ml were mixed with minimal sorbose medium and distributed over 10 plates.

For the induction of reversions with NA the method outlined under the heading "Induction of mutants by NA" was used. The suspensions were incubated at 25° C and stopped at the desired times, washed with pH 7 buffer solution and plated on minimal sorbose medium. Before and after treatment small samples were taken to determine the viability before treatment and the survival after treatment in the manner as described under the heading "Spontaneous reversion test."

RESULTS AND DISCUSSION

A. Induction of Tryptophan Auxotrophs

Eight experiments were carried out in which conidia of strain 74-A were used for the induction of tryptophan-dependent mutants and during which the filtration-enrichment method of WOODWARD, DE ZEEUW and SRB (1954) was employed for selecting of the mutants. In each experiment 1 × 10^8 spores were treated for 96 minutes at 25° C in a solution of 0.005 M $NaNO_2$ and at pH 4.6 (average survival 53%). From the 3750 colonies selected and tested out on minimal as well as on minimal medium supplemented with tryptophan only 12 completely tryptophan-dependent colonies (isolation numbers: 4, 17, 22, 33, 35, 46, 47, 56, 62, 63) and 8 incomplete (Leaky) tryptophan mutants were isolated. Hence this method did not work very efficiently under the conditions employed and it was decided to use the inositol-less method developed by LESTER and GROSS (1961). During each experiment 1 × 10^8 conidia were treated and 500 colonies tested for their tryptophan dependence. During six successful experiments the following numbers of mutants were obtained:

Experiment I: 3 (isolation numbers 69, 79, 81)

Experiment II: 2 (isolation numbers 90, 94)

Experiment III: 7 (isolation numbers 99, 100, 106, 118, 113, 112, 111)

Experiment IV: 13 (isolation numbers 119, 120, 123, 124, 126, 127, 131, 132, 133, 135, 136, 138, 140)

Experiment V: 7 (isolation numbers 141, 147, 148, 149, 150, 142, 143)

Experiment VI: 7 (isolation numbers 163, 191, 194, 195, 155, 162, 167)

Mutants 113, 112, 111, 143, 162 and 167 were found to be leaky and were not used in further experimental work.

B. Growth Requirements

The growth requirements of the 43 complete tryptophan mutants were tested on:

(1) minimal medium supplemented with anthranilic acid;

(2) minimal medium supplemented with indole; and

(3) minimal medium supplemented with tryptophan.

With regard to their growth requirements they could be classified into the following three groups:

Group A - those which can grow on minimal medium supplemented with anthranilic acid, indole or tryptophan
(Isolation numbers: 4, 22, 33, 35, 53, 54, 62, 69, 79, 81, 99, 106, 118, 163)

Group B - those which can grow on minimal medium supplemented with indole or tryptophan
(Isolation numbers: 56, 90, 94, 120, 140, 141, 147, 149, 194)

Group C - those which can grow on minimal medium supplemented with tryptophan
(Isolation numbers: 17, 46, 47, 63, 94, 100, 119, 123, 124, 126, 127, 131, 132, 133, 135, 136, 138, 148, 150, 191)

C. Linkage Determinations

In order to establish the linkage relationship of the above mutants, all of the mutants of groups B and C were crossed to a fl⁻-a strain grown on minimal crossing medium supplemented with 100 mg of l-tryptophan per liter. These crosses were carried out in petri dishes containing 20 ml of medium. The opposite mating types were inoculated at opposite poles of the plates and after 15 days of incubation at 22° C the perithecia started to eject ascospores. One hundred ascospores from each cross were isolated and put into small tubes containing complete medium. After isolation the ascospores were heat shocked at 60° C to break their dormancy and incubated at 30° C. After germination the cultures were scored for fluffy (aconidial) phenotype and tryptophan dependence. It was found that none of the mutants of group B showed linkage with fluffy whereas all the mutants of group C did. It is therefore clear that under the given circumstances no indole-utilizing tryp-3 mutant had been recovered. This might indicate that these indole-utilizing tryptophan mutants located in linkage group IIR occur at an extremely low frequency or that the method employed (NA induction) is selective for mutants of groups B and C only.

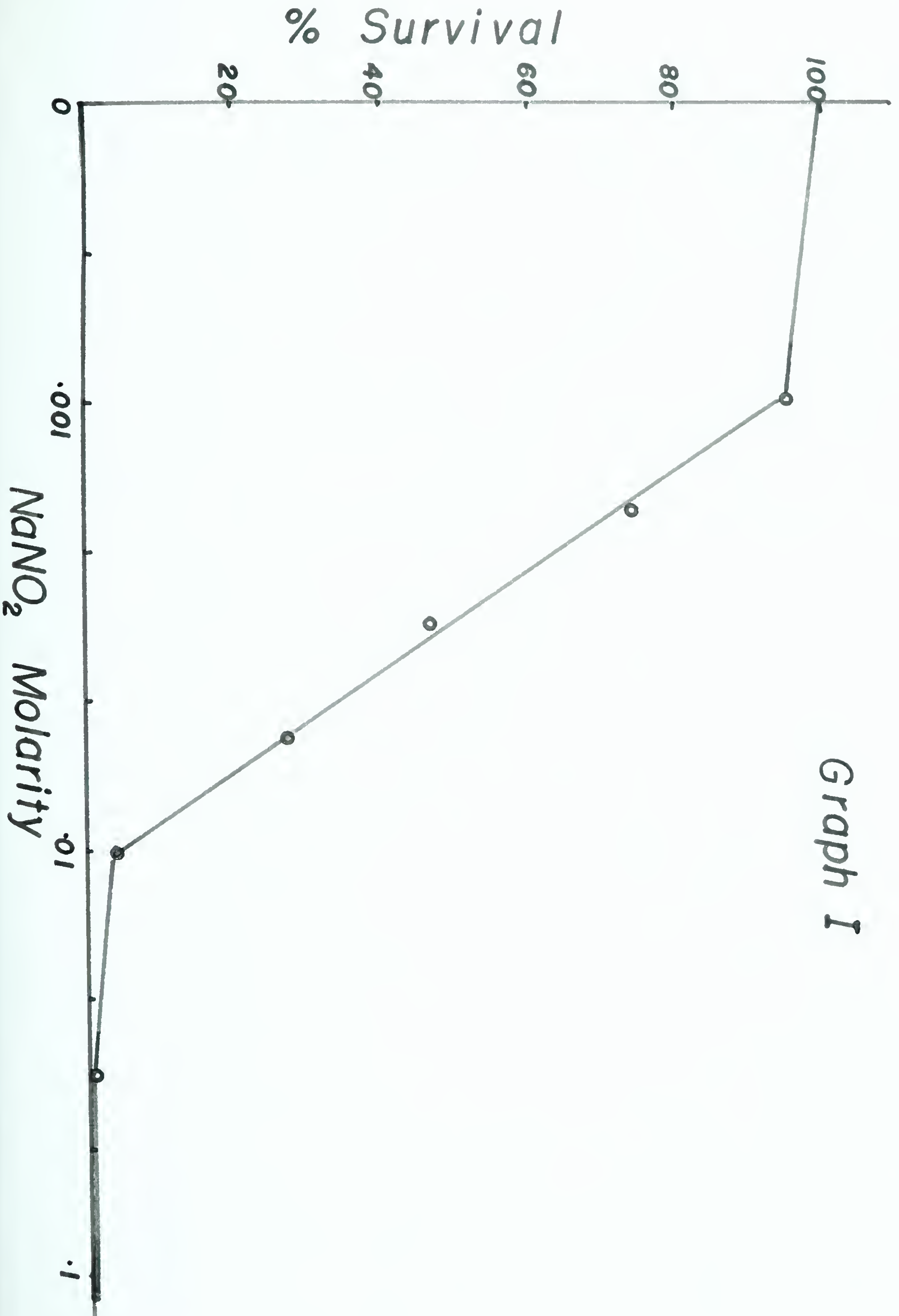
D. Inactivation

The process of inactivation of conidial spores was studied in order to determine the mode of inactivation of conidial spores by NA. The time of incubation for standard studies was arbitrarily set at 96 minutes. This long exposure was selected to give NA sufficient

time to penetrate the conidia and to react with the genetic material. Shorter exposure times were avoided in order to keep the NA concentration down, since it is known that NA alone deaminates proteins. In this latter instance conidial-kill would largely result from protoplasmic damage. Graph I, showing the survival of 74-A conidia suspended in various concentrations of NA for 96 minutes at pH 4.6 and at 25° C, shows that up to 0.001 M $NaNO_2$ there is insignificant inactivation; a linear relation between survival and $NaNO_2$ concentration is found between 0.001 M $NaNO_2$ and 0.01 M $NaNO_2$. Complete conidial kill is reached at 0.1 M $NaNO_2$. As a standard $NaNO_2$ molarity, 0.005 M was chosen, giving approximately 45 - 50% survival. Similar data were obtained for the inositol-requiring strain.

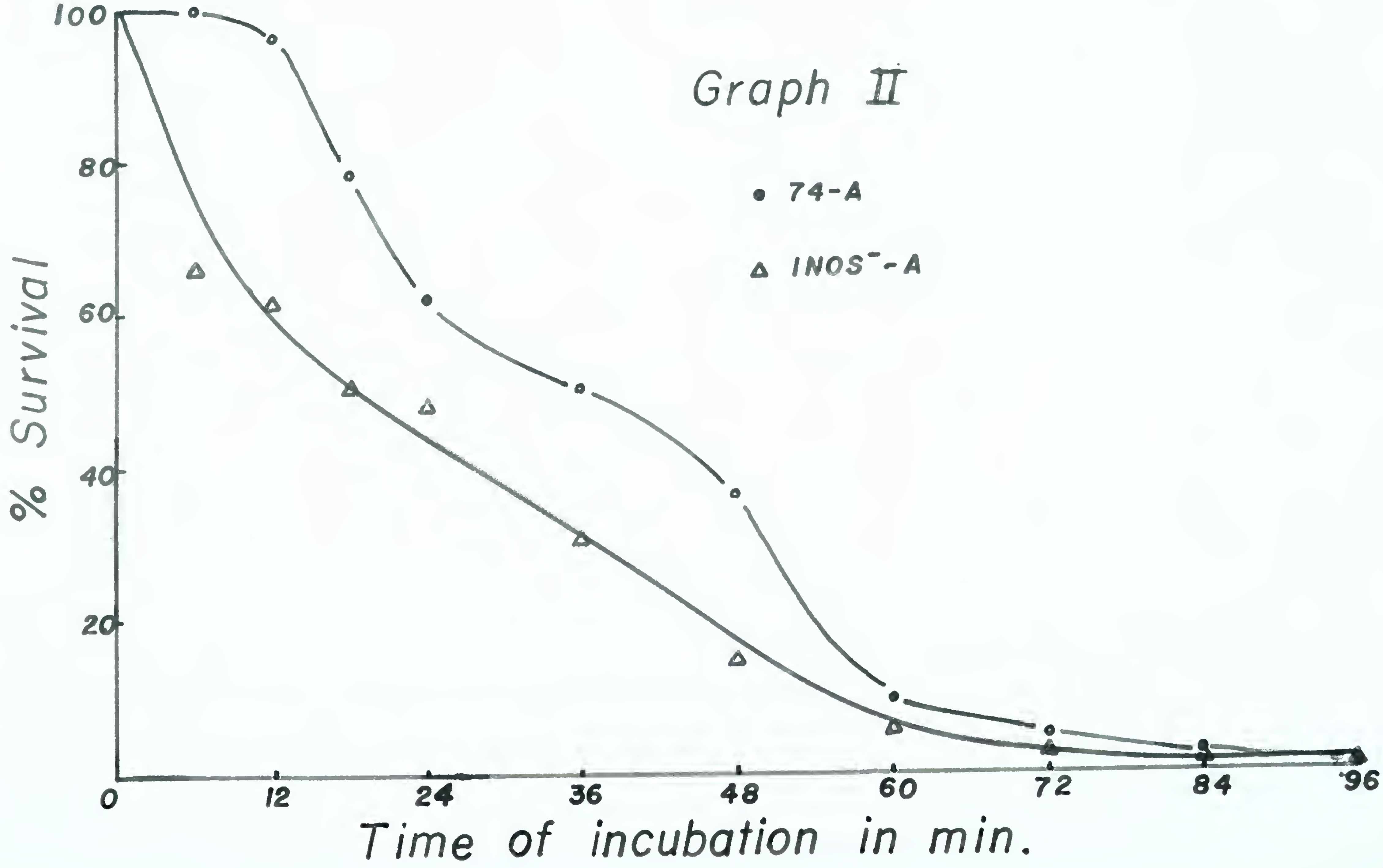
The rate of inactivation of conidia by 0.005 M NA was studied at 37° C as well as at 25° C. The results (the average of four experiments each) of the inactivation studies at 37° C for conidia of strain 74-A as well as for the inositol-requiring strain are shown in graph II. It can be noted that there exists a striking difference in the inactivation rates at the various times for the two strains tested. The inositol-requiring strain follows closely a single-hit inactivation curve whereas the survival of the conidia of the strain 74-A seems to be unaffected at short exposure times. The inactivation curve of strain 74-A shows a definite shoulder at short exposure times and in general follows an irregular pattern. The 37% survival times are also significantly different for both strains: 48 minutes for strain 74-A and 27 minutes for the inositol-requiring strain. The marked difference between the inactivation

Graph 1. The effect of $NaNO_2$ molarity on the survival of conidia of Neurospora crassa. Conidia of strain 74-A were incubated at 25° C in acetate buffer, pH 4.6, for 96 minutes. The suspension was adjusted to 1×10^7 per ml.



Graph I

Graph II. The effect of incubation time at 37° C on the survival of conidia of Neurospora crassa. Conidia of strains 74-A and inos$^-$ were incubated in acetate buffer, pH 4.6, and 0.005 M $NaNO_2$. The suspension was adjusted to 1×10^7 conidia per ml.


Graph II
● 74-A
△ INOS⁻-A
% Survival
100
80
60
40
20
0
12
24
36
48
60
72
84
96
Time of incubation in min.

of conidia of these strains can be explained by the differences in size of their conidia. On minimal medium 74-A hardly produces any micro-conidia (mononucleate) whereas the inositol-requiring strain gives rise to approximately 25% micro-conidia and to 75% macro-conidia (multinucleate). Also the average size of the macro-conidia of 74-A is about twice that of the average size of the inositol-requiring strain. It can be assumed that the rate of inactivation would be a function of:

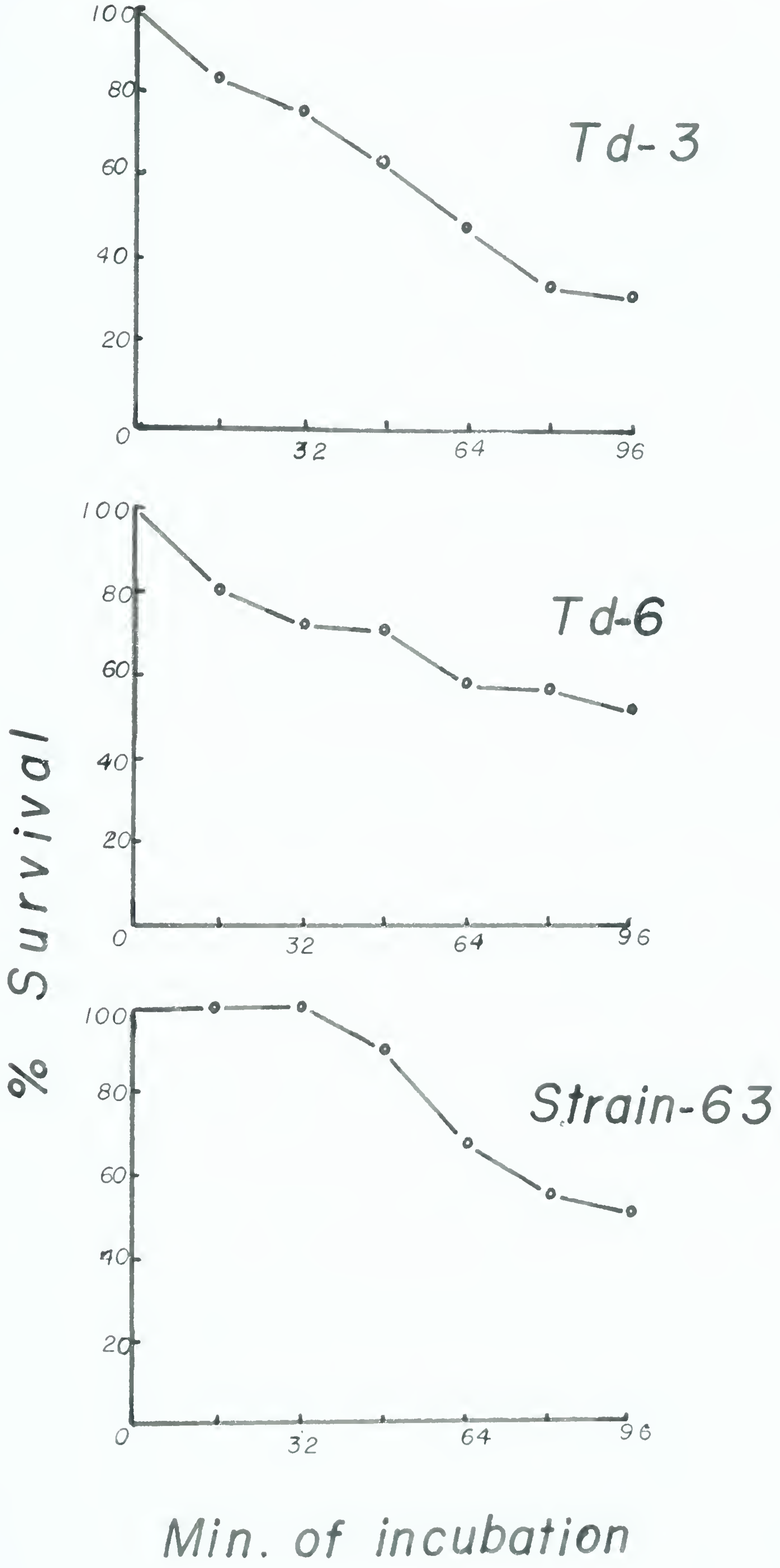
(i) the rate of penetration of NA;

(ii) the size of the conidium employed; and

(iii) the number of viable nuclei per conidium.

The data obtained (graph II) are in good agreement with these suppositions. However, inositol is also involved in the biochemistry of the cell wall. It seems reasonable to expect an impairment of cell-wall function and structure in those strains mutant for inositol synthesis. In other words such an impairment might also increase cell membrane permeability and ultimately result in quicker inactivation compared with strains autotrophic for inositol.

The inactivation curves for three different tryptophan mutants exposed to NA at 25° C are shown in graph III. Strain 63 was isolated during the course of the studies by exposing conidia of strain 74-A to NA. Strains td-6 and td-3 were obtained from the Fungal Genetic Stock Center and were initially induced by UV. Graph III shows the differences in inactivation rates between these strains. Strain 63 behaves like its parental strain 74-A at 37° C, except that at 25° C the rate of inactivation is slower, Graph III. The effect of incubation time at 25° C on the survival of conidia of Neurospora crassa. Conidia of strain 63, td-6 and td-3 were incubated in acetate buffer, pH 4.6, and 0.005 M $NaNO_2$. The suspension was adjusted to 1×10^7 conidia per ml.



Td-3
Td-6
Strain-63
% Survival
100
80
60
40
20
0
32
64
96
Min. of incubation

Graph III

causing the appearance of a longer "shoulder" in the inactivation curve. The curve obtained is typical for the cumulative, "multi-hit" type of action. Strain td-3 behaves like the inositol-requiring strain at 37° C. It is difficult to explain the single-hit kinetics of inactivation if this was due to genetic damage alone. KAUDEWITZ (1963) found multiple-hit kinetics for inactivation of *Escherichia coli* DNA, which is also in the haploid stage. It is possible that the single-hit inactivation curve observed is due to the complementary action of genetic and cytoplasmic damage of NA on the conidia.

E. Reversion Rates

Spontaneous reversion rates, as well as NA- and UV-induced reversion rate of the NA-induced mutants have been studied and the results of these studies are compiled in tables I, II, III and IV. The average reversion rates of the NA-induced mutants are as follows:

(1) spontaneous reversion: 1 per 6.3×10^6 conidia (maximum 1 per 1.3×10^6)

(2) NA-induced reversions: 1 per 1.5×10^6 conidia (maximum 1 per 2.2×10^5)

(3) UV-induced reversions: 1 per 4.7×10^5 conidia (maximum 1 per 9.8×10^4)

From these data the following ratio, between the various reversion rates can be calculated:

Spontaneous : NA : UV = 1 : 4.3 : 13.2.

Table 1. Spontaneous reversion frequencies for the NA-induced tryp-3 mutants

Strain	Viability (%)	Number x 10^7 viable conidia plated	Spontaneous reversion rate per 10^8 viable conidia plated, after 3 days at 30° C
17	87	36.0	14.8
46	92	8.5	6.3
47	83	45.0	27.0
63	72	11.0	9.0
94	83	8.8	0.0
100	92	16.0	77.0
119	91	11.0	leaky
123	71	12.0	39.0
124	86	15.0	21.3
126	81	10.0	29.0
127	83	15.0	3.4
131	84	11.0	24.8
132	94	11.0	2.7
133	62	6.7	1.3
135	77	19.0	8.4
136	75	18.0	1.2
138	92	14.0	5.0
148	85	8.5	4.7
150	86	8.8	6.8
191	87	15.0	leaky

Table II. Ultraviolet light induced reversion frequencies for the NA-induced tryp-3 mutants

Strain	Survival (%)	Number x 10^7 viable conidia plated	UV-induced reversion rate per 10^7 viable conidia plated, after 3 days at 30° C
17	47	0.47	46.8
46	44	0.53	1.8
47	50	0.5	2.0
63	57	0.57	58.0
94	52	0.57	63.2
100	52	0.52	111.0
123	41	0.47	7.3
124	49	8.0	3.6
126	46	9.6	3.7
127	52	1.0	5.5
131	50	5.5	5.0
132	57	5.7	12.3
133	45	0.45	17.7
135	32	0.36	5.5
136	47	0.47	19.1
138	40	0.4	3.7
148	51	0.5	0.0
150	49	0.49	23.4

Table III. Nitrous acid induced reversion frequencies for the NA-induced tryp-3 mutants

Strain	Survival (%)	Number x 10^7 viable conidia plated	UV-induced reversion rate per 10^7 viable conidia plated, after 3 days at 30° C
17	74	28.0	2.9
46	65	7.6	6.1
47	33	12.0	10.5
63	50	3.6	45.0
94	35	4.5	2.0
100	71	4.3	25.0
119	64	14.0	leaky
123	61	9.5	4.2
214	72	10.0	8.3
126	68	7.2	7.0
127	73	9.5	1.3
131	57	9.9	1.4
132	51	1.1	6.3
133	45	1.9	0.7
135	49	1.4	1.0
136	52	0.7	2.8
138	41	2.1	0.5
148	53	4.2	0.7
150	44	1.4	0.0
191	47	2.2	leaky

Table IV. Comparison of spontaneous, UV- and NA-induced reversion rates

Strain	Spontaneous reversion rate per 10^7 viable conidia	UV-induced reversion rate per 10^7 viable conidia	NA-induced reversion rate per 10^7 viable conidia	Ratio:		
				UV/S	NA/S	UV/NA
17	1.5	46.8	2.9	31.2	1.9	16.0
46	0.6	1.8	6.1	3.0	10.1	0.3
47	2.7	2.0	10.5	0.8	3.9	0.2
63	0.9	58.0	45.0	64.4	50.0	1.3
94	0.0	63.2	2.0	2.0	0.0	31.6
100	7.7	111.0	25.0	14.1	3.2	4.4
123	3.9	7.3	4.2	1.8	1.1	1.7
124	2.1	3.6	8.3	1.7	3.9	0.43
126	2.9	3.7	7.0	1.3	2.4	0.5
127	0.3	5.5	1.3	16.1	3.8	4.2
131	2.5	5.0	1.4	2.0	0.6	3.5
132	0.3	12.3	6.3	45.5	23.3	1.9
133	0.13	17.7	0.7	137.0	5.4	25.0
135	0.8	5.5	1.0	6.8	1.2	5.5
136	0.1	19.1	2.8	191.0	28.0	6.8
138	0.5	3.7	0.5	7.4	1.0	7.4
148	0.5	0.0	0.7	0.0	1.4	0.0
150	0.7	23.4	0.0	33.0	0.0	0.0

It is evident that the yield of reversions is greatly enhanced by the use of UV as a mutagen. The probable explanation for the effectiveness of UV may indicate that:

(i) NA induces a higher proportion of non-genetic hits (i.e. protoplasmic damage) per inactivation compared with UV; and

(ii) the mutants induced by NA have an altered base-pair sequence to which NA has a lower specificity for mutation when compared with UV.

For example the mutational base-pair changes $\begin{matrix} A \\ \| \\ T \end{matrix} \longleftrightarrow \begin{matrix} G \\ ||| \\ C \end{matrix}$ are known to be 6.3 times faster when going to the left than when going to the right, because *in vitro*, cytosine is deaminated 6.3 times faster than is guanine (VIELMETTER and SCHUSTER 1960). This indicates that most mutants induced by NA should have an A/T base pair at the mutant site and the mutants which show the highest reversion rates induced by NA should have a G/C base pair at the mutant site. When we compare NA-induced reversions with UV-induced reversions 4 out of 18 mutants (Table IV) are found to have higher NA-induced reversion rates when compared with the UV-induced reversion rates. In these instances the proportion of non-genic hits by UV is higher than for NA. In these instances a G/C base pair is involved at the mutant site rather than an A/T pair.

The high efficiency of UV as a reversion agent (when compared with NA) might be explained on the basis that all the mutants involved in the back-mutation tests were initially induced by NA. Since NA has a preference for deamination of the base C the majority of the mutants will have an A/T base pair at the mutational

site. As a result of a differential mutational mechanism (UV by excitation and NA by deamination) the A/T pair is more frequently attacked by UV than by NA, for which it is rather insensitive.

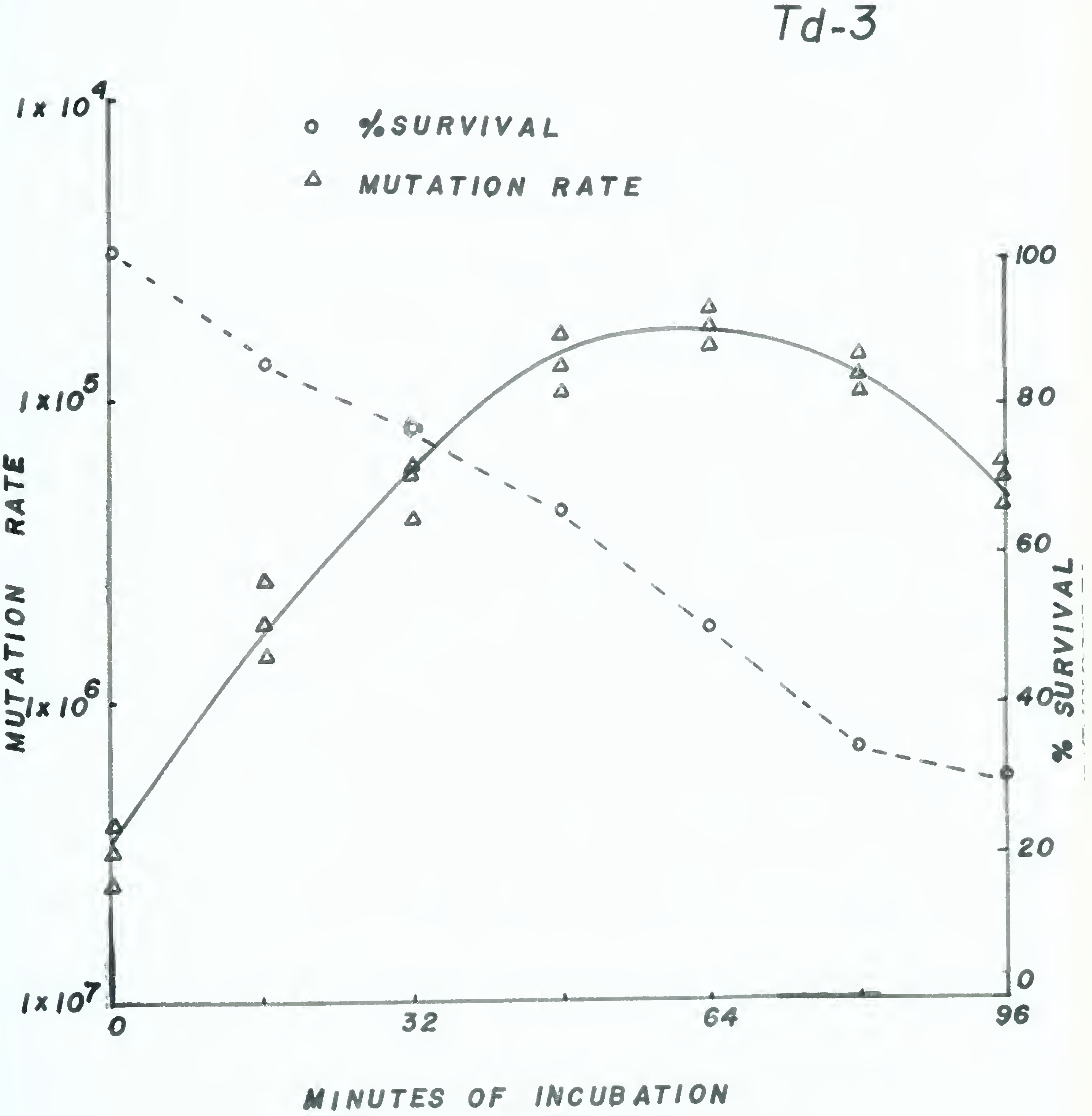
Another interesting observation can be made from Table IV. In the cases when the NA-induced reversion rates are higher than the UV-induced reversion rate we find that the UV-induced reversion rates are low for these strains, when they are compared with other mutant strains. We expect a G ≡ C base pair at these sites because of the relatively high NA-induced reversion rates. This could indicate that UV has a low efficiency for mutation of G/C base pairs and higher efficiency for the A = T base pairs.

F. Pattern of Reversion Induction

In preliminary experiments it was found that strains 63, td-3 and td-6 responded to NA-induction of back mutations (reversions). The dose-effect curves for the induction of reversions of the conidia of the above-named strains treated with NA at 25° C, according to the method described earlier, are shown in graphs IV, V and VI. The background spontaneous revertants have been subtracted from the observed reversion rate and the resulting NA-induced reversion rates have been expressed as the average number of viable conidia showing one reversion. The graphs show that the strains differ largely in their back-mutation rates and in their survival patterns. However, the dose-effect curves for the induction of reversions are similar. They all show their optimum reversion rate at around 64 minutes of treatment. This could indicate that the differences in the survival

Graph IV. The effect of time of incubation in NA on (a) survival and (b) back-mutation rate of conidia of strain td-3 of *Neurospora crassa*. The conidia were incubated at 25° C in acetate buffer, pH 4.6, and 0.005 M $NaNO_2$. The suspension was adjusted to 1×10^7 conidia per ml.

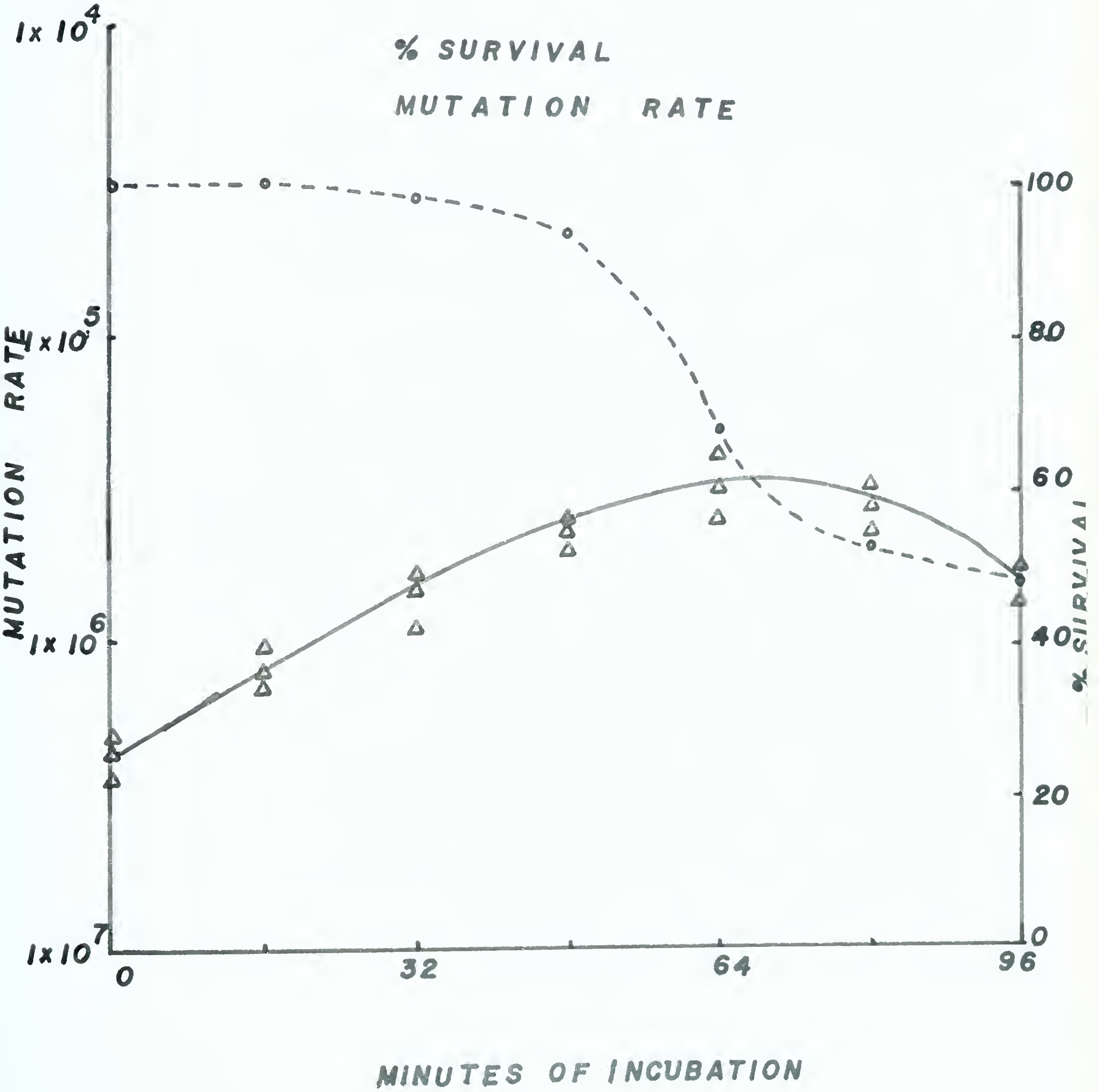
Graph IV


Td-3
o %SURVIVAL
Δ MUTATION RATE
1 x 10⁴
1 x 10⁵
1 x 10⁶
1 x 10⁷
MUTATION RATE
100
80
60
40
20
0
% SURVIVAL
0
32
64
96
MINUTES OF INCUBATION

Graph V. The effect of time of incubation in NA on (a) survival and (b) back-mutation rate of conidia of strain 63 of *Neurospora crassa*. The conidia were incubated in acetate buffer, pH 4.6, and 0.005 M $NaNO_2$. The suspension was adjusted to 1×10^7 conidia per ml.

Graph V

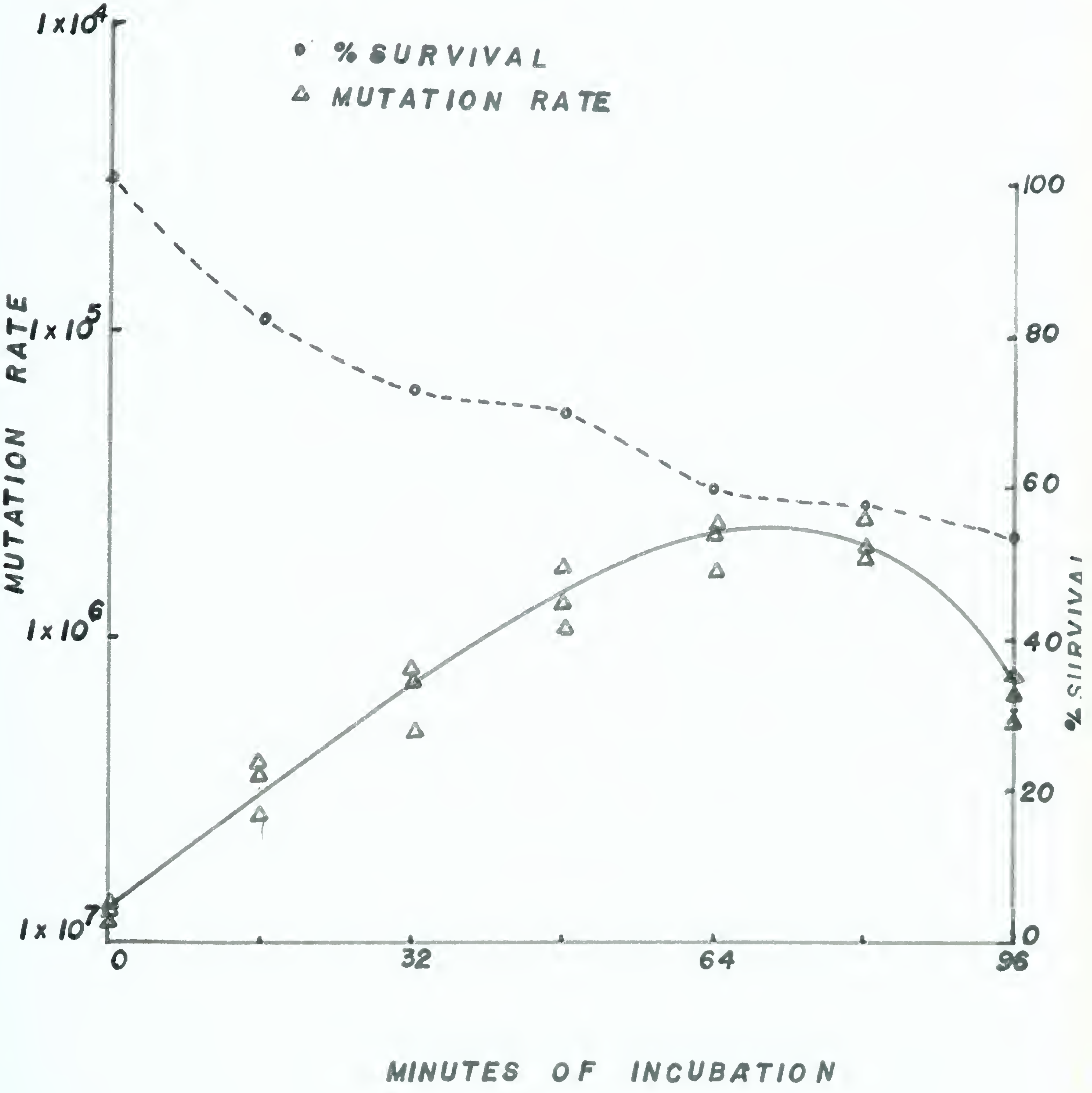
Strain-63



Graph VI. The effect of time of incubation in NA on (a) survival and (b) back-mutation rate of conidia of strain td-6 of Neurospora crassa. The conidia were incubated at 25° C in acetate buffer, pH 4.6, and 0.005 M $NaNO_2$. The suspension was adjusted to 1×10^7 conidia per ml.

Graph VI

Td-6


• % SURVIVAL
△ MUTATION RATE
MUTATION RATE
1 x 10^4
1 x 10^5
1 x 10^6
1 x 10^7
100
80
60
40
20
0
% SURVIVAL
0
32
64
96
MINUTES OF INCUBATION

rates found, in these strains at least, are due to cytoplasmic rather than due to the genetic damage by NA. If they are due to genetic damage we would also expect a difference in the dose-effect curves for the induction of reversions. The observation that the maximum number of reversions were found at 64 minutes for the conditions employed in this study means that it would have been more accurate to determine the NA-induced reversion rates after 64 minutes of treatment with NA than after the 96 minutes employed. Strain td-3 shows an approximately 16-fold increase in the NA-induced reversion rate at the optimum dose found. The survival of the treated conidia at that dose was 50%. This means that an absolute increase in the frequency of back mutations due to the treatment of NA has been obtained. This indicates that NA acts as a mutagenic agent for conidia of _N_. _crassa_ rather than as a selective agent for the mutations already present in the treated conidia.

SUMMARY

The induction of auxotrophic mutants, and the inactivation of conidia of *Neurospora crassa* by nitrous acid (NA) has been studied. Also the reversion rates in NA-induced tryp-3 mutants by NA and ultra-violet light (UV) have been studied.

The conidial kill was found to vary with the strains employed. Strain 74-A showed a cumulative type of inactivation curve, while an inositol-requiring strain showed a single-hit inactivation curve. The differences in inactivation between these strains were thought to be due to the differences in cytoplasmic damage, rather than to differences in genetic damage.

The dose-effect curves for the induction of reversions in three strains of tryp-3 mutants were found to be non-linear with time and showed a definite optimum time of incubation under the conditions employed. A sixteen-fold increase in the reversion frequency by NA-treatment was found at 50% inactivation. This means an absolute increase of reverse mutation by NA treatment was found, indicating that NA is a mutagenic agent for conidia of *Neurospora crassa*. The NA- and UV-induced reversion rates obtained for these initially NA-induced tryp-3 mutants were found to be in agreement with the rate and kind of base-pair changes as found by other workers.

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